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Abstract: The toxicity of six different Planktothrix strains was examined in acute toxicity assays with the crustacean Thamnocephalus platyurus. The presence of toxicity in two strains could be explained by the occurrence of microcystins. The other four Planktothrix strains were not able to produce microcystins due to different mutations in the microcystin synthetase (mcy) gene cluster. In these strains, toxicity was attributed to the presence of chlorine and sulfate containing compounds. The main representative, called aeruginosin 828A, of such a compound in the Planktothrix strain 91/1 was isolated, and structure elucidation by 2D NMR and MS methods revealed the presence of phenyllactic acid (Pla), chloroleucine (Cleu), 2-carboxy-6-(4 -sulfo-xylosyl)-octahydroindole (Choi), and 3-aminoethyl-1-N-amidino-Δ-3-pyrroline (Aeap) residues. Aeruginosin 828A was found to be toxic for T. platyurus with a LC50 value of 22.4 M, which is only slightly higher than the toxicity found for microcystins. Additionally, very potent inhibition values for thrombin (IC50 = 21.8 nM) and for trypsin (IC50 = 112 nM) have been determined for aeruginosin 828A. These data support the hypothesis that aeruginosins containing chlorine and sulfate groups, which were found in microcystin-deficient Planktothrix strains, can be considered as another class of toxins.

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The toxicity and enzyme activity of a chlorine and sulfate containing aeruginosin isolated from a non-microcystin-producing *Planktothrix* strain

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ABSTRACT:

The toxicity of six different *Planktothrix* strains was examined in acute toxicity assays with the crustacean *Thamnocephalus platyurus*. The presence of toxicity in two strains could be explained by the occurrence of microcystins. The other four *Planktothrix* strains were not able to produce microcystins due to different mutations in the microcystin synthetase (*mcy)* gene cluster. In these strains, toxicity was attributed to the presence of chlorine and sulfate containing compounds. The main representative, called aeruginosin 828A, of such a compound in the *Planktothrix* strain 91/1 was isolated, and structure elucidation by 2D-NMR and MS methods revealed the presence of phenyllactic acid (*Pla*), chloroleucine (*Cleu*), 2-carboxy-6-(4'-sulfo-xylosyl)-octahydroindole (*Choi*), and 3-aminoethyl-1-*N*-amidino-Δ-3-pyrroline (*Aeap*) residues. Aeruginosin 828A was found to be toxic for *Thamnocephalus platyurus* with a LC_{50} value of 22.4 µM, which is only slightly higher than the toxicity found for microcystins. Additionally, very potent inhibition values for thrombin (IC_{50} = 21.8 nM) and for trypsin (IC_{50} = 112 nM) have been determined for aeruginosin 828A. These data support the hypothesis that aeruginosins containing chlorine and sulfate groups, which were found in microcystin-deficient *Planktothrix* strains, can be considered as another class of toxins.

Keywords: microcystin, *mcy* gene cluster, inhibitor, toxin, microcystin-deficient

1. **Introduction**

Climate change as well as the anthropogenic input of nutrients into freshwaters has resulted in an increase of cyanobacterial biomasses in many lakes and rivers throughout the world during the last decades (Paerl and Huisman, 2008). These organisms are often found to accumulate in very high densities also in lakes that are intensively used as recreational areas or as drinking water reservoirs (Posch et al., 2012). However, cyanobacteria are known to produce and store intracellularly a wide variety of bioactive secondary metabolites, and a collapse of such a bloom might liberate high amounts of these compounds into the water. In recent years, substantial progress has been made to identify possible harmful compounds from different cyanobacterial genera. Most of these substances could be assigned to distinct chemical oligopeptide classes, e.g. microcystins, cyanopeptolins, anabaenopeptins, aeruginosins, cyclamids, microginins and microviridins (Welker and von Döhren, 2006). These peptides exhibit various biological activities such as the inhibition of different phosphatases and proteases (Welker and von Döhren, 2006). Microcystins (MCs) have been found to be responsible for countless animal poisonings all over the world, and even for human toxicity (Sivonen and Jones, 1999), and cyanopeptolins and aerucyclamides received some attention due to their toxicity to *Thamnocephalus platyurus* (Blom et al., 2003; Gademann et al., 2010; Portmann et al., 2008a; Portmann et al., 2008b).

There is intense discussion about the possible ecological role of MCs in the environment; however, MCs are still regarded to be the primary defence mechanism of cyanobacteria against grazers (Blom et al., 2001; Kurmayer and Jüttner, 1999). MC-deficient *Planktothrix* genotypes are typically found in rather low proportions of the total cyanobacterial population (Ostermaier and Kurmayer, 2009), but might occasionally exceed 50% of the total abundances. Reasons for the lack of MC production might be the complete loss of or diverse mutations (e.g. insertions or deletions) within the MC synthetase (*mcy*) gene cluster (Christiansen et al., 2006; Christiansen et al., 2008). However, the loss or dysfunction of the *mcy* gene cluster did not seem to be disadvantageous for the overall success of the *Planktothrix* genotypes. This is supported by the linear relation of MC deficient genotypes to

the total population density of bloom forming *Planktothrix* populations in European lakes (Ostermaier and Kurmayer, 2009). In order to explain these findings it was suggested that an alternative peptide or peptide class might functionally compensate for the lack of MCs. Here we report on a peptide class, the chlorine and sulfate containing aeruginosins, which were found in MC-deficient strains, and which exhibited acute toxicity against the crustacean *T. platyurus*.

2. Material and methods

2.1. General Experimental Procedures.

^{1}H-NMR spectra were recorded on a Bruker Avance III 600 MHz with a 5 mm BBFO plus probe or a Bruker Avance III Ascend 700 MHz spectrometer with a 5 mm TCI (H-C/N-D) cryo probe at room temperature. Chemical shifts (δ-values) are reported in ppm, spectra were calibrated related to the solvent residual proton chemical shift (DMSO, δ = 2.50). The coupling constants are specified in Hz. HRMS spectra were recorded on a Bruker maXis 4G instrument. Identification and purification of aeruginosin 828A (**1**) was performed on a Shimadzu 10AVP HPLC system equipped with an automated sample injector, a thermostated column compartment, and photo diode array detector. Mass spectra were recorded on a combined LC-MS (LCQ Duo mass spectrometer, Finningan Thermoquest, USA) equipped with an electrospray ionization source (ESI-MS).

2.2. Culture, Extraction, and Bioassay guided fractionation.

A culture collection of six *Planktothrix* strains was established in the laboratory at the beginning of this study. All cyanobacteria were grown in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 µmol m^{-2} s^{-1} from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000K) in 120 mL mineral medium described by Jüttner and co-workers (Jüttner et al., 1983). Two of the six *Planktothrix* strains were capable of producing MCs, four strains lacked the MC production either due to complete loss *of* or due mutations *within* the MC synthethase (*mcy*) gene cluster (summarized in Table S1, Supplementary

material). The analysis of the *mcy* gene cluster in *Planktothrix* strains was carried out as part of another study as described elsewhere (Christiansen et al., 2008).

Frozen biomass of all six *Planktothrix* strains was extracted twice with 50 % MeOH (10 mL per gram of wet cell biomass) for 2 h in the dark. After centrifugation (25'700 g for 15 min) the supernatants ('crude extracts') were fractionated by HPLC equipped using a reversed phase column (Hydrosphere C18, YMC, 4.6 x 250 mm, Stagroma, Reinach, CH) using two solvents: UV-treated deionised water and acetonitrile. The solvents were free of trifluoroacetic acid (TFA) to avoid undesired toxic side effects and unwanted isomerisation products (Blom et al., 2001). A linear increase was applied (acetonitrile from 20 % to 70 % in 50 min, 70 to 100 % in 2 min, isocratic for additional 10 min). Fractions were collected every three minutes; the solvents were evaporated by vacuum centrifugation (SPEEDVAC Plus® SC110A, Savant Instruments Inc. USA). The fractions containing the different cyanobacterial compounds were transferred to a 24 well plate, and re-dissolved in 1 % MeOH (500 µL). The toxicity of the fractions of the six crude extracts was tested in a 24-h acute toxicity assay performed using instar II-III larvae (Thamnotoxkit F; MicroBioTests Inc.) of the sensitive crustacean *Thamnocephalus platyurus*, which can be easily hatched from cysts (Blom et al., 2003; Blom et al., 2001). About 20 to 30 crustaceans were transferred to each well; after 24 h, the mortality rate was determined for each fraction. Each fraction represented the extract of an equivalent of 0.05 µM chlorophyll a of the biomass of the six *Planktothrix* strains.

2.3. Isolation of aeruginosin 828A.

Fresh biomass (32 g) of *Planktothrix* strain 91/1 was extracted with 50 % MeOH. Crude extracts were obtained after centrifugation and were separated by HPLC using a reversed phase column (Hydrosphere C18, YMC, 4.6 x 250 mm, Stagroma, Reinach, CH) under the following conditions: solvent A was UV-treated deionised water (+ 0.05 % TFA), solvent B: HPLC-grade acetonitrile (+ 0.05 % TFA); a linear increase was applied (as described above). Under the conditions applied aeruginosin 828A (**1**) eluted after 16.0 min. TFA was removed before evaporating the solvents to prevent undesirable isomerization products by applying

sequentially the combined HPLC fractions on a C18 cartridge (10 g; Mega Bond Elute, Varian, Agilent Technologies, Basel, CH; conditioned with 10 % MeOH). The cartridge was flushed with water to remove the TFA, and aeruginosin 828A (**1**) was eluted with 80 % MeOH. Subsequently, the aqueous methanol was evaporated (35 mbar, 40 °C) to achieve a colourless, amorphous solid. After purification 533 µg of pure aeruginosin A828 (> 99 % HPLC) could be achieved.

Aeruginosin 828A (**1**): UV (47% acetonitrile in water with 0.05 % TFA λ_{max} 277 nm; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; HRMS-ESI: calcd. for $C_{36}H_{53}{}^{35}ClN_6O_{12}SNa^+$ $[M+Na]^+$: 851.3023; found: 851.3019.

2.4. Acute toxicity of aeruginosin 828A.

The highly purified aeruginosin 828A was tested in a 24 h acute toxicity assay with *T. platyurus* in six concentrations ranging from 0.5 to 100 µM in triplicates. For every concentration, 20 to 30 animals were used. The nonlinear regression analysis as well as the LC_{50} value were calculated using Graph Pad Prism 5 for Windows.

2.5. Enzyme inhibition assays.

The inhibition of trypsin (0.04 U/ 200 µL; No 9471 Fluka, Buchs, Switzerland), and thrombin (5 nM; IHTa Innovative Research, Peary, USA) was tested in microtiter plates. Boc-Gln-Ala-Arg-aminomethylcoumarin (50 µM; Bachem AG, Bubendorf, Switzerland) served as substrate for trypsin, and Boc-Phe-Ser-Arg- aminomethylcoumarin (100 µM; Bachem AG, Bubendorf, Switzerland) as substrate for thrombin according to previously established protocols (Blom et al., 2006; Gademann et al., 2010). All fluorescent substrates were dissolved in H_2O (5% DMSO). The reaction solution consisted of 140 µL Tris buffer (50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1 mg/mL BSA), 10 µL enzyme solution, 30 µL toxin solution, and 20 µL fluorescent substrate; samples were measured at 37 °C for 20 min in a Fluorescence plate reader (SpectraMAX, GeminiXS, Molecular Devices Corp., USA) with the excitation wavelength at 380 nm and the emission wavelength at 440 nm.

158 ## 3. Results and Discussion

159 *3.1. Bioassay-guided fractionation*

160 Methanolic extracts of the biomasses of six *Planktothrix* strains (Fig. 1) were
161 fractionated and assayed against the sensitive freshwater crustacean *T. platyurus* for acute
162 toxicity. Overall, more than 35 compounds were present in these fractions, and out of total 72
163 fractions, twelve were found to be highly toxic. In these fractions, three compounds were
164 assigned to known MCs, seven to unknown (sulfate-containing) cyanopeptolins, and ten were
165 sulfate and chlorine containing peptides of the size of aeruginosins (Fig. 1).

166

167 *3.2. Structure elucidation of aeruginosin 828A*

168 In order to elucidate the structure of the unknown toxin, the major toxic oligopeptide of
169 *P. rubescens* strain 91/1 was purified and analysed by NMR spectroscopy. The putative
170 molecular ions at *m/z* = 851.3019 $[M+Na]^+$ and *m/z* = 853.3003 $[M+Na]^+$ in the HRMS spectrum
171 support a molecular formula of $C_{36}H_{53}{}^{35}ClN_6O_{12}SNa^+$ or of $C_{36}H_{53}{}^{37}ClN_6O_{12}SNa^+$ for the
172 respective Na adduct. In addition, the mass spectrum suggests the presence of a sulfate group
173 by the fragment with *m/z* = 749, as well as the presence of one Cl atom with its characteristic
174 isotope pattern (Fig.S1, Supplementary material). The ^{1}H–NMR spectrum of aeruginosin 828A
175 (**1**) (Table 1; Fig. S2 Supplementary material) displays two sets of characteristic peptide
176 resonances, among others. The constitution of each of the building blocks was assigned using
177 COSY, HSQC, HMBC and NOESY experiments, and the chemical shifts as well as key HMBC
178 and NOE correlations are reported in Table 1. These 2D-NMR experiments and comparison
179 to literature data led to the identification of the following building blocks: phenyllactic acid (Pla);
180 chloroleucine (Cleu); 2-carboxy-6-(4'-sulfo-xylosyl)-octahydroindole (Choi) fragment and 3-
181 aminoethyl-1-*N*-amidino-Δ3-pyrroline residue (Aeap). The position of the Cl atom in Cleu was
182 assigned based on the characteristic chemical shift of the corresponding Cβ atom of the Leu
183 moiety (δ_c = 68.6 ppm). The presence of a carbohydrate residue could be detected due to the
184 low field shifted carbon atoms ($\delta_c \approx 70$ ppm), which indicated the presence of oxygen
185 substituents. The xylose was identified based on the typical proton and hydroxy group

substitution pattern. The position of the sulfate group was established by the typical chemical shift of Xyl C-4 (δ_c = 74.7 ppm), by the COSY correlations between H-2 (δ_H = 3.28 ppm) and Xyl OH-2 (δ_H = 4.41 ppm), as well as between Xyl H-3 (δ_H = 3.57 ppm) and Xyl OH-3 (δ_H = 4.96 ppm) (Table 1; Fig. S3 and S4, Supplementary material). The relative configuration of the carbohydrate moiety was assigned on the basis of the NOESY spectrum and *J*-coupling constants. The α-anomer of the sugar moiety was assigned by its small coupling constant (*J* = 3.8 Hz), which results from the (+)-*sc* arrangement between Xyl H-1 to Xyl H-2. The configuration of the Choi moiety was assigned using the relevant NOE correlations between Choi H-3' (δ_H = 1.81 ppm) and Choi H-4' (δ_H = 1.48 ppm), as well as Choi H-4 (δ_H = 2.14 ppm) and Choi H-3a (δ_H = 2.25 ppm), Choi H-3 (δ_H = 2.01, 1.81 ppm) to Choi H-3a (δ_H = 2.25 ppm) and, additionally, to Choi H-6 (δ_H = 3.83 ppm) and Choi H-7' (δ_H = 2.25 ppm). The configuration at the C-6 stereogenic center of Choi was assigned on the basis of the line width of its signal (total ~ 16 Hz), which implies that no trans-diaxial relationship of the Choi H-6 to its coupling partners is present. Due to the lack of commercially available, authentic standards for the Cleu and Choi residues, we did not perform hydrolysis and subsequent analysis on chiral stationary phases. The observed NOE between Choi H-3a and Choi H-7a supports the relative *cis*-configuration, which is further substantiated by numerous literature examples of identical configurations. The tentatively assigned relative configuration of aeruginosin 828A (**1**) is further corroborated by comparison with data reported for the structurally similar aeruginosin 205B (Table S2, Supplementary material) (Hanessian et al., 2009). This natural aeruginosin differs only by an agmatine moiety instead of the Aeap residue and shows almost identical ^{1}H and ^{13}C shifts. We therefore assign the relative configuration for the Pla and Cleu building blocks as shown for compound **1**. Yet, we are aware that its final structure elucidation will require the use of total chemical synthesis.

The assembly of the different fragments was established by HMBC and NOESY experiments (Fig.S5 and S6, Supplementary material). The linkage between central Choi and the Cleu was established by a HMBC correlation between Choi H-7a (δ_H = 4.32 ppm) and Cleu C-1 (δ_c = 167.4 ppm). In a similar fashion, the connection between the Choi and the Aeap

residues was determined by the coupling between Aeap H-1 and H-1' (δ_H = 3.16 ppm and 3.23 ppm) and Choi C-1 (δ_c = 171.1 ppm). The HMBC correlation from Cleu H-2 (δ_H = 4.93 ppm) to Pla C-1 (δ_c = 172.5 ppm) unambiguously demonstrated the connection of these moieties. In addition, the connection of the Xylose unit with the Choi moiety was elucidated by the NOE correlation of the Xyl H-1 (δ_H = 4.94 ppm) to Choi H-6 (δ_H = 3.83 ppm) as well as by the HMBC correlation of the Xyl H-1 (δ_H = 4.94 ppm) to Choi C-6 (δ_c = 68.4 ppm).

3.3. Protease inhibitory activities

Aeruginosin 828A (**1**) belongs to a large group of linear peptides that feature the (hydroxyl)phenyllactic acid (Hpla/Pla) or glyceric acid at the N-terminus (position 1), followed by a variable amino acid in position 2, the Choi moiety in position 3, and an arginine derivative at the C-terminus in position 4 (Welker and von Döhren, 2006). These peptides have been evaluated for high inhibitory activity against trypsin and trypsin-like serine proteases such as thrombin. Aeruginosin 828A (**1**) exhibited strong inhibition towards these proteases: an IC_{50} value of 21.8 nM was measured for thrombin, an IC_{50} value of 112 nM for trypsin (Fig. 1). Thus, aeruginosin 828A (**1**) is one of the strongest inhibitors of trypsin-like enzymes among the aeruginosins, together with chlorodysinosin A, and the aeruginosins 205A and B (Hanessian et al., 2006; Shin et al., 1997; Toyooka et al., 2003). Common to these four aeruginosins is the presence of a Cleu in position 2 that was so far unknown for natural products (Hanessian et al., 2006), and of either a sulfated glyceric acid derivative at the N-terminus or a sulfated Choi subunit. A comparison of the biological activity of other aeruginosins led to the hypothesis that both, chlorine and sulfate moieties are necessary for strong inhibition and toxicity of these peptides.

It was proposed that the Choi sulfate group would increase the selectivity for trypsin rather than for thrombin (Sandler et al., 1998), and that the sulfate group in the N-terminal position would make a significant contribution upon inhibiting thrombin (Carroll et al., 2004). This is supported by the hydrogen bonding network of the dysinosin A-thrombin complex (Carroll et al., 2002). The N-terminated sulfate group of dysinosin A interacted with arginines

208 and 263 of the thrombin with several hydrogen bonds, whereas the 5,6-dihydroxyoctahydroindole group did not appear to have any hydrogen bonding interactions with thrombin. A structure and activity comparison of different aeruginosins revealed that the inhibition values for trypsin of Choi sulfated aeruginosins were lower than the IC_{50} values for thrombin and that the presence of the N-terminal sulfate group considerably decreased the IC_{50} values for thrombin (Table 2, groups 2 and 3). The IC_{50} values for the inhibition of trypsin are typically in the low micromolar range. However, for a strong inhibition of thrombin in the low nanomolar range, the N-terminal sulfate group seems to be essential (Table 2, groups 2 and 3). The inhibition values for aeruginosin 828A seem to be in opposition to this more general trend. However, other factors, such as the arginine derivative in position 4 might have an additional influence on the inhibition potential towards trypsin-like proteases as it was shown for the guanidino group of dysinosin A that strongly bonded to aspartic acid 229 of thrombin (Carroll et al., 2002). Future experiments by using derivatives of aeruginosin 828A might address this conundrum.

The absence of chlorine was thought to be responsible for the comparatively high concentration of aeruginosin 98B that was required for plasmin and thrombin inhibition (Sandler et al., 1998). However, the comparison of different aeruginosins revealed that a chlorine at the N-terminus does not significantly contribute to the inhibition of trypsin-like proteases (Table 2, groups 2 and 4). Only the chlorine of Cleu in position 2 might indicate an additional positive effect upon inhibiting proteases such as trypsin and thrombin, as indicated by the lowest IC_{50} values for these aeruginosins (Table 2, group 1).

3.4. Toxicity of aeruginosin 828A

Aeruginosins have been long known for their inhibition potential towards trypsin-like serine-proteases, however, toxicity was not described to date (Ersmark et al., 2008; Nagarajan et al., 2013). Assessment of acute toxicity of aeruginosin 828A (**1**) towards *T. platyurus* revealed a LC_{50} value of 22.4 µM. This value is comparable to those determined for MCs, e.g. a LC_{50} value of 10.8 µM was measured for MC-LR (Blom and Jüttner, 2005). This clearly shows

that aeruginosin 828A (**1**) represents another toxin in *P. rubescens* strain 91/1, a strain that has lost the ability to produce microcystins due to an insertion in the *mcy* gene cluster. Such toxicity might be also unveiled for other aeruginosins. Bioassay-guided fractionations showed strong toxicity of aeruginosins only, if they contain both, chlorine and sulfate (Fig. 1). Aeruginosins bearing only sulfate, or containing neither sulfate nor chlorine did not seem to be toxic to *T. platyurus* in the concentrations tested (*P. rubescens* strain 79, fraction 2; *P. rubescens* strain 91/1, fraction 3; Fig. 1).

Water-bloom forming cyanobacteria such as *Planktothrix* are producing several bioactive substances, most prominently MCs that are the most abundant toxins in freshwater. Besides the fact that MCs strongly inhibit the protein phosphatases 1 and 2A, the ecological function of MCs is still not clear. However, it is widely agreed that MCs reduce significantly the survival rate of grazers such as crustaceans (Blom et al., 2006; Kurmayer and Jüttner, 1999; Rohrlack et al., 1999). Blooms of cyanobacteria are typically composed of microcystin- and non-microcystin-producing strains that evolved independently (Christiansen et al., 2008). In this study, we investigated the toxicity of aeruginosin 828A that was found in a MC deficient strain. However, MCs and aeruginosins clearly inhibit a different spectrum of enzymes pointing to a different mode of action. In future, it will be necessary to consider not only the MCs but also compounds such as aeruginosin 828A to forecast the toxicity of cyanobacterial blooms and to unravel their impact on various aquatic organisms.

4. Conclusions

In this note, we have reported the structure elucidation of aeruginosin 828A (**1**) isolated from *P. rubescens* strain 91/1, which inhibits trypsin and thrombin in the low nanomolar range. Additionally, aeruginosin 828A (**1**) was found to be toxic for the freshwater crustacean *T. platyurus*, and its toxicity was determined to be only slightly higher than MCs that are the prototypical examples of peptide toxins in cyanobacteria. This report thus suggests the presence of alternative toxins in MC-deficient *Planktothrix* strains and encourages further evaluation of the presence and toxicity of modified aeruginosin variants in *Planktothrix* strains.

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SUPPLEMENTARY MATERIAL AVAILABLE:

The NMR spectra, key NOE correlations and additional data for aeruginosin 828A (**1**) are available free of charge via the Internet at http://pubs.acs.org.

308 **REFERENCES:**

309 Blom, J.F., Baumann, H.I., Codd, G.A., Jüttner, F., 2006. Sensitivity and adaptation of
310 aquatic organisms to oscillapeptin J and [D-Asp^3,(*E*)Dhb^7]microcystin-RR. Arch. Hydrobiol.
311 167(1-4), 547-559.

312 Blom, J.F., Bister, B., Bischoff, D., Nicholson, G., Jung, G., Süssmuth, R.D., Jüttner, F.,
313 2003. Oscillapeptin J, a new grazer toxin of the freshwater cyanobacterium *Planktothrix*
314 *rubescens*. J. Nat. Prod. 66(3), 431-434.

315 Blom, J.F., Jüttner, F., 2005. High crustacean toxicity of microcystin congeners does not
316 correlate with high protein phosphatase inhibitory activity. Toxicon 46(4), 465-470.

317 Blom, J.F., Robinson, J.A., Jüttner, F., 2001. High grazer toxicity of [D-
318 Asp^3,(*E*)Dhb^7]microcystin-RR of *Planktothrix rubescens* as compared to different
319 microcystins. Toxicon 39(12), 1923-1932.

320 Carroll, A.R., Buchanan, M.S., Edser, A., Hyde, E., Simpson, M., Quinn, R.J., 2004.
321 Dysinosins B-D, inhibitors of factor VIIa and thrombin from the Australian sponge
322 Lamellodysidea chlorea. J. Nat. Prod. 67(8), 1291-1294.

323 Carroll, A.R., Pierens, G.K., Fechner, G., de Almeida Leone, P., Ngo, A., Simpson, M., Hyde,
324 E., Hooper, J.N.A., Bostrom, S.L., Musil, D., Quinn, R.J., 2002. Dysinosin a: A novel inhibitor
325 of factor VIIa and thrombin from a new genus and species of Australian sponge of the family
326 dysideidae. J. Am. Chem. Soc. 124(45), 13340-13341.

327 Christiansen, G., Kurmayer, R., Liu, Q., Börner, T., 2006. Transposons inactivate
328 biosynthesis of the nonribosomal peptide microcystin in naturally occurring Planktothrix spp.
329 Appl. Environ. Microbiol. 72(1), 117-123.

330 Christiansen, G., Molitor, C., Philmus, B., Kurmayer, R., 2008. Nontoxic strains of
331 cyanobacteria are the result of major gene deletion events induced by a transposable
332 element. Mol. Biol. Evol. 25(8), 1695-1704.

333 Elkobi-Peer, S., Faigenbaum, R., Carmeli, S., 2012. Bromine- and chlorine-containing
334 aeruginosins from *Microcystis aeruginosa* bloom material collected in Kibbutz Geva, Israel. J.
335 Nat. Prod. 75(12), 2144-2151.

336 Ersmark, K., Del Valle, J.R., Hanessian, S., 2008. Chemistry and biology of the aeruginosin
337 family of serine protease inhibitors. Angew. Chem.-Int. Edit. 47(7), 1202-1223.

338 Gademann, K., Portmann, C., Blom, J.F., Zeder, M., Jüttner, F., 2010. Multiple toxin
339 production in the cyanobacterium *Microcystis*: isolation of the toxic protease inhibitor
340 cyanopeptolin 1020. J. Nat. Prod. 73(5), 980-984.

341 Hanessian, S., Del Valle, J.R., Xue, Y.F., Blomberg, N., 2006. Total synthesis and structural
342 confirmation of chlorodysinosin A. J. Am. Chem. Soc. 128(32), 10491-10495.

343 Hanessian, S., Wang, X.T., Ersmark, K., Del Valle, J.R., Klegraf, E., 2009. Total synthesis
344 and structural revision of the presumed aeruginosins 205A and B. Org. Lett. 11(18), 4232-
345 4235.

346 Ishida, K., Okita, Y., Matsuda, H., Okino, T., Murakami, M., 1999. Aeruginosins, protease
347 inhibitors from the cyanobacterium Microcystis aeruginosa. Tetrahedron 55(36), 10971-
348 10988.

349 Jüttner, F., Leonhardt, J., Möhren, S., 1983. Environmental factors affeting the formation of
350 mesityloxide, dimethylallylic alcohol and other volatilve compounds excreted by Anabaena
351 cylindrica. J. Gen. Microbiol. 129(FEB), 407-412.

352 Kurmayer, R., Jüttner, F., 1999. Strategies for the co-existence of zooplankton with the toxic
353 cyanobacterium Planktothrix rubescens in Lake Zurich. J. Plankton Res. 21(4), 659-683.

354 Matsuda, H., Okino, T., Murakami, M., Yamaguchi, K., 1996. Aeruginosins 102-A and B, new
355 thrombin inhibitors from the cyanobacterium *Microcystis viridis* (NIES-102). Tetrahedron
356 52(46), 14501-14506.

357 Murakami, M., Ishida, K., Okino, T., Okita, Y., Matsuda, H., Yamaguchi, K., 1995.
358 Aeruginosin 98-A and B, trypsin inhibitors from the blue-green alga Microcystis aeruginosa
359 (NIES-98). Tetrahedron Lett. 36(16), 2785-2788.

360 Nagarajan, M., Maruthanayagam, V., Sundararaman, M., 2013. SAR analysis and bioactive
361 potentials of freshwater and terrestrial cyanobacterial compounds: a review. J. Appl. Toxicol.
362 33(5), 313-349.

363 Ostermaier, V., Kurmayer, R., 2009. Distribution and abundance of nontoxic mutants of
364 cyanobacteria in lakes of the Alps. Microb. Ecol. 58(2), 323-333.

365 Paerl, H.W., Huisman, J., 2008. Climate - Blooms like it hot. Science 320(5872), 57-58.

366 Portmann, C., Blom, J.F., Gademann, K., Jüttner, F., 2008a. Aerucyclamides A and B:
367 Isolation and synthesis of toxic ribosomal heterocyclic peptides from the cyanobacterium
368 *Microcystis aeruginosa* PCC 7806. J. Nat. Prod. 71(7), 1193-1196.

369 Portmann, C., Blom, J.F., Kaiser, M., Brun, R., Jüttner, F., Gademann, K., 2008b. Isolation of
370 aerucyclamides C and D and structure revision of *Microcyclamide* 7806A: Heterocyclic
371 ribosomal peptides from *Microcystis aeruginosa* PCC 7806 and their antiparasite evaluation.
372 J. Nat. Prod. 71(11), 1891-1896.

373 Posch, T., Köster, O., Salcher, M.M., Pernthaler, J., 2012. Harmful filamentous
374 cyanobacteria favoured by reduced water turnover with lake warming. Nat. Clim. Change 2,
375 809-813.

376 Rohrlack, T., Dittmann, E., Henning, M., Börner, T., Kohl, J.G., 1999. Role of microcystins in
377 poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium
378 *Microcystis aeruginosa*. Appl. Environ. Microbiol. 65(2), 737-739.

379 Sandler, B., Murakami, M., Clardy, J., 1998. Atomic structure of the trypsin-aeruginosin 98-B
380 complex. J. Am. Chem. Soc. 120(3), 595-596.

381 Shin, H.J., Matsuda, H., Murakami, M., Yamaguchi, K., 1997. Aeruginosins 205A and -B,
382 serine protease inhibitory glycopeptides from the cyanobacterium Oscillatoria agardhii (NIES-
383 205). J. Org. Chem. 62(6), 1810-1813.

384 Sivonen, K., Jones, G., 1999. Cyanobacterial toxins, In: Chorus, I., Bartram, J. (Eds.), Toxic
385 cyanobacteria in water. E. and F.N. Spon, London and New York, pp. pp. 41-111.

386 Toyooka, N., Nakazawa, A., Himiyama, T., Nemoto, H., 2003. Synthesis of the N-terminus of
387 glycopeptide unit for aeruginosin 205-A. Heterocycles 59(1), 75-79.

388 Welker, M., von Döhren, H., 2006. Cyanobacterial peptides - Nature's own combinatorial
389 biosynthesis. Fems Microbiol. Rev. 30(4), 530-563.
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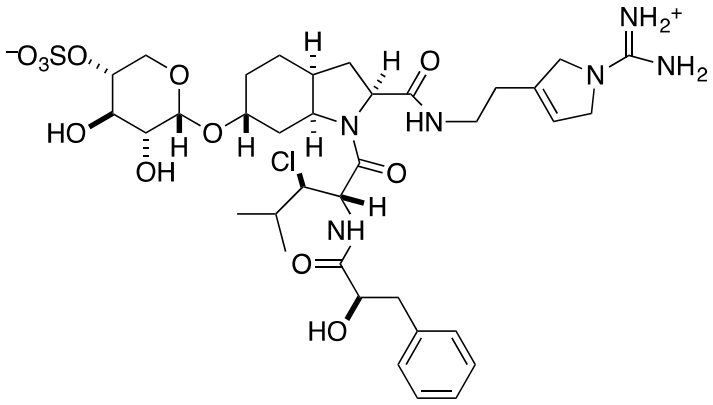
391
392

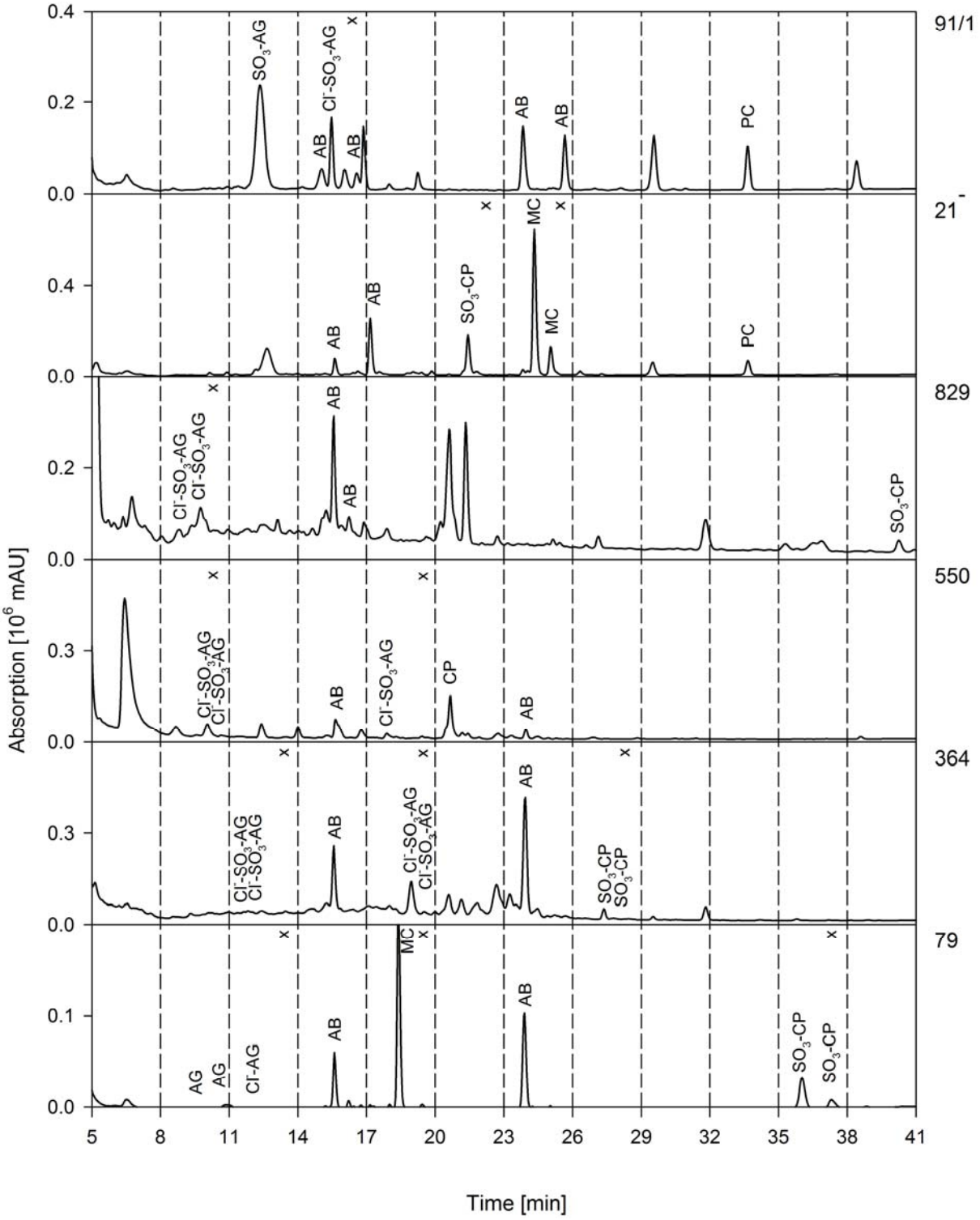
FIGURES & LEGENDS

Fig. 1. HPLC-chromatograms (220 nm; 0.05 µM chlorophyll equivalent) of all six *Planktothrix* strains (AB = Anabaenopeptin, AG = Aeruginosin, Cl^--AG = Cl^--Aeruginosin, Cl^--SO_3-AG = Cl^--SO_3-Aeruginosin, CP = Cyanopeptolin, SO_3-CP = SO_3-Cyanopeptolin, MC = Microcystin, PC= Planktocyclin; x = fraction was toxic to *T. platyurus*).

Fig. 2. Trypsin and thrombin inhibition curves of aeruginosin 828A (**1**).

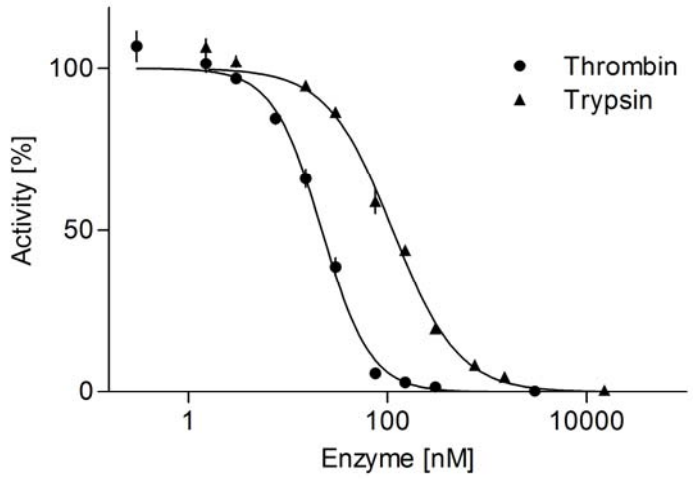


Aeruginosin 828A (**1**)



410

411 **Fig. 1.** HPLC-chromatograms (220 nm; 0.05 µM chlorophyll equivalent) of all six *Planktothrix*
412 strains. (AB = Anabaenopeptin, AG = Aeruginosin, Cl^--AG = Cl^--Aeruginosin, Cl^-SO_3-AG = Cl^-
413 -SO_3-Aeruginosin, CP = Cyanopeptolin, SO_3-CP = SO_3-Cyanopeptolin, MC = Microcystin, PC=
414 Planktocyclin; x = fraction was toxic to *T. platyurus*).
415



416

417 **Fig. 2.** Trypsin and thrombin inhibition curves of aeruginosin 828A (**1**).

418

419

420 **Table 1**. NMR spectroscopic data for aeruginosin 828A.

421 **Aeruginosin 828A** (700MHz, DMSO-d6, 298K)

422

Residue	Position	^{1}H (*J* in Hz)	^{13}C (from 2D)	HMBC[a]	NOE
Xyl	1eq	4.94 d (3.8)	95.0	Xyl 2,3,5, Choi 6	Choi 6, Xyl 2,4',7,7'
	2ax	3.28 m	71.8	Xyl 3,5	Xyl 1,4
	3ax	3.57 ddd (2.6, 9.0, 9.1)	71.4	Xyl 2,4,5	Xyl 4,5ax, 3-OH
	4ax	3.93 ddd (5.8, 9.1, 10.6)	74.7	Xyl 3,5	Xyl 1,2,5ax,5eq,3-OH
	5ax	3.36 dd (10.6, 10.7)	59.3	Xyl 1,3,4	Xyl 4,5eq
	5eq	3.67 dd (5.8, 10.7)		Xyl 1,3,4	Xyl 4,5ax
	2-OH	4.41 (7.3)	-	Xyl 1,2	-
	3-OH	4.96 (2.6)	-	Xyl 2,3,5	Xyl 3,4
Choi	1	-	171.1	-	-
	2	4.18 dd (9.4, 8.2)	59.5	Choi 1,3	Aeap NH, Choi 3,3',3a,7
	3	2.01 ddd (12.6, 7.3, 7.3)	30.5	Choi 3a,7a	Choi 2,3a,3',4',6,7',7a
	3'	1.81 ddd (12.6, 12.6, 9.7)	-	Choi 1,2,3a	Choi 2,3,3a,4',5',7
	3a	2.25 m	35.6	Choi 3,4,5,7,7a	Choi 2,3',4',7,7a
	4	2.14 m	19.1	Choi 3,3a,5	Choi 3a,4',5,5'
	4'	1.48 m	-	Choi 5,6,7a	Choi 3a,4,5',6
	5	1.49 m	24.4	Choi 6,7a	Choi 6,Cleu 2
	5'	1.54 m	-	-	Choi 3a,4,5,6,7'
	6	3.83 m	68.4	-	Choi 5,5',7,7', Xyl 5ax
	7	1.58 dd (11.9, 12.9)	28.4	Choi 7a	Cleu 2, Choi 6,7'
	7'	2.25 m	-	Choi 5,7a	Choi 2,6,7',7a, Cleu 2
	7a	4.32 ddd (11.9, 6.4, 6.4)	54.1	Choi 2,3,3a,7, Cleu 1	Choi 3a,4,4',7,7', Cleu 2,5
Pla	1	-	172.5	-	-
	2	4.18 (br)	71.5	-	Pla 3, 3'
	3	2.79 dd (14.0, 7.6)	39.7	Pla 1,2,4,5,9	Pla 2,3'
	3'	2.96 dd (14.0, 3.7)	-	Pla 1,2,4,5,9	Pla 2,3
	4	-	137.9	-	-
	5,9	7.23 m	129.5	Pla 3,5,7,9	Pla 3,3',7
	6,8	7.26 m	127.7	Pla 4,6,8	Pla 5,7,9
	7	7.18 m	125.8	Pla 5,9	-
	2-OH	-	-	-	-
Cleu	1	-	167.4	-	-
	2	4.93 dd (10.7,8.7)	50.9	Cleu 1,3, Pla 1	Cleu 3,4,5, Choi 7a,7,7'
	3	4.00 dd (10.7, 1.8)	68.6	Cleu 1,2,4,5,5'	Cleu 2,4,5'
	4	1.71 dsept. (6.6, 1.8)	27.3	Cleu 5,5'	Cleu 2,3,5
	5	0.87 3H, d (6.6)	15.3	Cleu 3,4,5'	Cleu 2,3,4,l Choi 7',7a
	5'	0.86 3H, d (6.6)	20.6	Cleu 3,4,5	Cleu 2,3,4,l Choi 7',7a
	NH	7.68 d (8.7)	-	-	Cleu 3
Aeap	1	3.16 dddd (13.0, 6.5, 6.5, 5.7)	36.3	Aeap 2,3, Choi 1	Aeap 2,4,5,6,NH, Choi 2
	1'	3.23 dddd (13.0, 6.5, 6.5, 5.7)	-	Aeap 2,3, Choi 1	Aeap 2,4,5,6,NH, Choi 2
	2	2.25 2H, m	28.1	Aeap 1,3,4,6	Aeap 1,1',4,5,6,NH
	3	-	136.1	-	-
	4	5.61 t (1.6)	119.0	Aeap 2,3,5,6	Aeap 1,1',2,5,6,NH
	5	4.07 2H, d (1.6)	53.6	Aeap 3,4	Aeap 1,1',2,4
	6	4.07 2H, s	54.9	Aeap 3,4	Aeap 1,1',2,4
	NH	8.00 dd (5.7, 5.7)	-	Aeap 1, Choi 1	Aeap 1,1',2,4,5,6, Choi 2,3,3'
	8	-	154.9	-	-

[a]HMBC correlations are given from proton(s) stated to the indicated carbon atom.

423

Table 2. Structure characteristics and enzyme inhibition of aeruginosins (that are characterized by the following structure: Pla/Hpla/Glyceric acid – Amino acid – Choi – Arg derivative).

Group	Aeruginosin example (number of peptides evaluated)		Modifications (Cl, chlorination; Br, bromination; SO_3, sulfation)			Enzyme inhibition (IC_{50}) [7]	
			Pla/Hpla/ Glyceric acid	Amino acid	Choi	Trypsin [μM]	Thrombin [μM]
1	Chlorodysinosin A[1]	(1)	SO_3	Cleu	---	0.037	0.0057
	Aeruginosin 828A[2]	(3)	---	Cleu	SO_3	0.09-0.112	0.022-1.9
2	Aeruginosin 89A[3]	(2)	Cl, SO_3	Leu	---	0.6-9.2	0.04-0.07
	Aeruginosin 98A[4]	(6)	Cl, Cl_2, Br, Br_2	Ile	SO_3	0.1-18.2	3.6- >45.5
3	Aeruginosin 102-A[5]	(6)	SO_3	Tyr / Val / Leu	---	0.27-1.5	0.05-0.14
	Aeruginosin 98B[4]	(1)	---	Ile	SO_3	0.9	15.3
4	Aeruginosin GE 686[6]	(5)	Cl, Cl_2, Br, Br_2	Ile/Leu	---	2.2-8.5	12.8- >45.5

[1] (Hanessian et al., 2006); [2] (This study); [3] (Ishida et al., 1999); [4] (Murakami et al., 1995); [5] (Matsuda et al., 1996); [6] (Elkobi-Peer et al., 2012); [7] IC_{50} values reported in mg mL^{-1} were converted to μM.